The altered human serum metabolome induced by a marathon

Abbreviated title: Altered metabolome induced by a marathon

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ABSTRACT

Introduction: Endurance races have been associated with a substantial amount of adverse effects which could lead to chronic disease and long-term performance impairment. However, little is known about the holistic metabolic changes occurring within the serum metabolome of athletes after the completion of a marathon.

Objectives: Considering this, the aim of this study was to better characterize the acute metabolic changes induced by a marathon.

Methods: Using an untargeted two dimensional gas chromatography time-of-flight mass spectrometry metabolomics approach, pre- and post-marathon serum samples of 31 athletes were analyzed and compared to identify those metabolites varying the most after the marathon perturbation.

Results: Principle component analysis of the comparative groups indicated natural differentiation due to variation in the total metabolite profiles. Elevated concentrations of carbohydrates, fatty acids, tricarboxylic acid cycle intermediates, ketones and reduced concentrations of amino acids indicated a metabolic shift between various fuel substrate systems. Additionally, elevated odd-chain fatty acids and α-hydroxy acids indicated the utilization of α-oxidation and autophagy as alternative energy-producing mechanisms. Adaptations in gut microbe-associated markers were also observed and correlated with the metabolic flexibility of the athlete.

Conclusion: From these results it is evident that a marathon places immense strain on the energy-producing pathways of the athlete, leading to extensive protein degradation, oxidative stress, mammalian target of rapamycin complex 1 inhibition and autophagy. A better understanding of this metabolic shift could provide new insights for optimizing athletic performance, developing more efficient nutrition regimens and identify strategies to improve recovery.

Keywords: marathon; serum; metabolomics; metabolite markers; fuel substrates

1. INTRODUCTION

Although physical activity has been shown to be substantially beneficial to human health (Ojiambo, 2013), various negative effects including cardiovascular dysfunction (Webner *et al.*, 2012), muscle damage (Howatson *et al.*, 2010), increased propensity to upper respiratory tract infection (Robson-Ansley *et al.*, 2012) and severe inflammation (Bonasia *et al.*, 2015) have been associated with running extensive distances, such as that of endurance races. Even though these races have become increasingly popular, limited research is based on the elucidation of the effects of these races using a metabolomics approach. Metabolomics is defined as the identification and quantification of the small metabolite compounds (<1500 Da) present in a biological system in order to determine the physiological effects induced by a specific perturbation (Heaney *et al.*, 2017). Since metabolites are typically the end-products of the genome, transcriptome and proteome, alterations in these are indicative of the overall physiological state of the investigated biological system (Heaney *et al.*, 2017).

Previous metabolomics studies have indicated elevated concentrations of various carbohydrate/glycolysis metabolite intermediates, indicative of free glucose utilization as the preferred energy source during strenuous physical activity (Lewis *et al.*, 2010; Salway, 2012; Waśkiewicz *et al.*, 2012). Furthermore, significant alterations to the tricarboxylic acid cycle intermediates were induced by a marathon (Turer *et al.*, 2014) and could be attributed to additional strain placed on the electron transport chain (ETC), causing an imbalanced NADH:NAD^+ ratio (Esterhuizen *et al.*, 2017). According to previous work (Stellingwerff, 2012), free glucose and other carbohydrate stores can become depleted within approximately 90 min after the start of the race, which most likely lead to the utilization of alternative fuel substrates (lipids and amino acids) for energy production (Waśkiewicz *et al.*, 2012). Increased lipolysis activity results in elevated serum glycerol and free fatty acids (Lewis *et al.*, 2010; Waśkiewicz *et al.*, 2012), the latter of which produce acetyl-CoA via β-oxidation and subsequent energy via the TCA cycle and ETC (Salway, 2012). Furthermore, the increased synthesis of acetyl-CoA could also ascribe the elevated ketone concentrations previously reported (Pechlivanis *et al.*, 2010), as it is a key component of ketogenesis. In the event that the strenuous physical activity continues beyond the capacity of the athlete's lipid stores, or if the traditional lipid oxidation pathways become saturated

(Staron *et al.*, 1989), the athlete's metabolism shifts towards protein catabolism (resulting in reduction blood amino acid levels) in an attempt to synthesize the energy required to complete the marathon (Lewis *et al.*, 2010). These amino acids are primarily oxidized to pyruvic acid and acetyl-CoA, both of which can serve as TCA cycle influx substrates for energy production (Salway, 2012). Additionally, protein degradation has been shown to alter purine catabolism, resulting in elevated adenosine-monophosphate, inosine-monophosphate, hypoxanthine, xanthine, uric acid and allantoin (Turer *et al.*, 2014), the latter of which is a uric acid derivative and a surrogate index of oxidative stress (Lewis *et al.*, 2010).

Although these metabolomics studies provide some clues to the metabolic alterations that occur during strenuous physical activity such as long-distance endurance races, very few of these employed an untargeted metabolomics approach. Considering this, an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach was used to holistically compare the serum metabolite profiles of 31 recreational marathon athletes before and after the completion of a marathon (42 km), in order to better characterize the acute metabolic changes induced by exercise stress.

2. MATERIALS AND METHODS

2.1 Participants

All participants completed a health and dietary questionnaire (including a menstrual cycle questionnaire for female participants) prior to the marathon to assess their eligibility. Individuals with food allergies, cardiovascular complications, musculoskeletal disorders/injuries, or those receiving anti-inflammatory treatment were excluded from the study. Athlete participation in this investigation was completely voluntary, and all the participants gave written and informed consent. A summary of the participant characteristics is presented in Table 1.

2.2 Clinical samples

Blood samples were collected by antecubital fossa venesection of 31 marathon athletes (19 males and 12 females) 24 hours before and immediately after completing the Druridge Bay Marathon (Northumberland, UK). Pre-marathon samples were collected the day preceding the race (between 10am and 6pm) as a means of reducing additional metabolic changes induced by the venesection stress as well as to limit interference to the athletes' pre-marathon regimens. The individuals were required to be in a hydrated, yet fasted state (for a minimum of two hours) at time of baseline sample venesection. The samples were collected in standard 10 mL vacutainer vials, placed on ice and transported to the laboratory (Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation at the Northumbria University in Newcastle upon Tyne, UK) for immediate processing. Briefly, the blood was allowed to clot for 30 min and centrifuged at 3 000 *g* for 10 min. The supernatant (serum) was then extracted and immediately frozen (-80°C) before being transported (on dry ice) to the North-West University, Human Metabolomics: Laboratory of Infectious and Acquired Diseases. These serum samples were stored at -80°C until metabolomics analyses commenced.

2.3 Chemicals and reagents

Methoxamine hydrochloride, 3-phenylbutyric acid and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma Aldrich (St. Louis, Missouri, USA) and the pyridine was from Merck (Darmstadt, Germany). The acetonitrile used, was an ultra-pure Burdick and Jackson brand (Honeywell International Inc., Muskegon, Michigan, USA) and hence did not require any further purification.

2.4 Sample extraction and derivatization

A total metabolome extraction procedure was performed on all the collected serum samples, along with appropriate quality control (QC) samples. During this process 50 µL of internal standard, 3-phenylbutyric acid (0.45 µg/mL) dissolved in a chloroform:methanol:water (1:3:1) solution, was added to 50 µL of each serum sample. Hereafter, the samples were placed on ice before 300 µL of acetonitrile was added as a protein precipitation agent. Samples were then subjected to the REAX D-91126 vortex (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) for 2 min and centrifuged

at 3 500 *g* for 10 min at 4°C. The supernatant of the biphasic solution was then transferred to a clean GC-MS sample vile and dried at 40ºC under a light stream of nitrogen gas for approximately 45 min. Hereafter, 25 µL of methoxamine hydrochloride dissolved in pyridine (15 mg/mL) was added to each sample and incubated at 50°C for 90 min. Thereafter, 40 µL BSTFA with 1% TMCS was added, followed by derivatization for 60 min at 60°C. Each derivatized sample was transferred to a new GC-MS vile containing a vile insert and capped.

2.5 GCxGC-TOFMS analysis and processing

The derivatized samples were placed on a multi-purpose auto-sampler tray (Gerstel GmbH and co. KG, Mülheim van der Ruhr, Germany) in a randomized order, and analyzed using a Pegasus 4D GCxGC-TOFMS system (LECO Africa (Pty) Ltd, Johannesburg, South Africa), fitted with an Agilent 7890A GC and TOFMS (LECO Africa). During analyses, 1 µL of each sample was injected using a 1:3 split ratio. Purified helium was used as a carrier gas and set at a constant flow rate of 1 mL/min, while the injector temperature was set to operate at a constant 270°C throughout the entire sample analysis. The primary oven was fitted with a Restek Rxi-5MS capillary column (30 m; 0.25 µm diameter and 0.25 µm film thickness) and programmed to start the run at an initial temperature of 70°C for 2 min, followed by an increase of 4°C/min until a final temperature of 300°C was reached and maintained for 2 min. The secondary oven, equipped with a Restek Rxi-17 capillary column (1 m; 0.25 µm diameter and 0.25 µm film thickness), was programmed with an initial 85°C, which was increased by 4.5°C/min until a final temperature of 300°C was reached and maintained for 2 min. The thermal modulator was set to pulse streams of cold and hot nitrogen gas every 3 s, for 0.5 s. The detector was set to disregard all mass spectra (ms) information for the first 400 s of each run to exclude solvent detection; however, this was still included on the time axis of the primary column to reflect accurate retention times. Additionally, the transfer line and ion source were respectively held at a constant of 270°C and 220°C for the entire run, with a detector voltage of 1600 V and filament bias of -70 eV. Ms were acquired at a rate of 200 ms per second, over a range of 50–800 m/z. The total run time per sample was 111.28 min. Following GCxGC-TOFMS analysis, 838 peaks were identified, which was processed using LECO Corporation's ChromaTOF software (version 4.32), as described by (Luies and Loots, 2016).

2.6 Statistical analyses

Prior to statistical analysis, the data were normalized in relation to the internal standard and subjected to several "clean-up steps", including a 50% zero filter, QC drift correction, QC coefficient of variation filter and zero value replacement (Fernandez *et al.*, 2000; Luies and Loots, 2016). Hereafter, a natural shifted log transformation was performed to correct for skewed variable distribution, as well as auto scaling to align all variables (Van den Berg *et al.*, 2006) (exclusively during multivariate analysis).

Both multivariate and univariate statistical analyses were performed using MATLAB software (2012), adjusted with the PLS toolbox (2016), to identify the metabolite markers best describing the variation between the comparative groups. Multivariate approaches included principle component analysis (PCA) to determine whether a natural differentiation occurred between the comparative groups, and partial least square–discriminant analysis (PLS-DA) to characterize the group separation (Luies and Loots, 2016). The univariate data analyses included a Wilcoxon signed rank test, corrected for multiple testing by the Benjamini–Hochberg procedure, to assess the statistical significance of each compound (Benjamini and Hochberg, 1995; Rosner *et al.*, 2006), while an independent effect size calculation (Wilcoxon signed effect size) was used to assess the practical significance of each detected metabolite (Tomczak and Tomczak, 2014).

3. RESULTS

The PCA differentiation between serum metabolite profiles (Fig. 1) of the marathon athletes before and after the completion marathon was clearly defined. The total amount of variance explained by the first three principle components (PCs) (R_2X cum) was 53%, of which PC1 accounted for 24%, PC2 for 20% and PC3 for 9%. Additionally, the PLS-DA model (results not shown) showed a modelling parameter R_2Y (cum) of 48.81%, indicating the total variance of the response Y, and a Q_2 (cum) of 83.37%, indicating the cross-validation variation due to the response Y.

Since the aim of this study was to attain a holistic view of the altered human serum metabolome induced by a marathon, compounds with a PCA power value ≥ 0.5 or Wilcoxon p-value ≤ 0.017 (BH-critical

value) or an effect size ω-value ≥ 0.3 were considered significant and interpreted based on their associated metabolic/biochemical pathways. This multi-statistical approach yielded an initial list of 78 metabolite markers, of which 70 metabolite markers could be annotated by comparison of their mass spectra and retention times to that of commercially available and in-house libraries developed from previously injected standards (see Table 2).

4. DISCUSSION

The altered metabolite markers listed in Table 2 are indicative of the major metabolic pathways affected by the marathon and are mainly associated with the macro-fuel substrate utilization pathways (carbohydrates, lipids and amino acids) and the regulation thereof (TCA, oxidative phosphorylation [OXPHOS] and gut microbiome). These metabolite pathways, along with the intermittent dietary-associated metabolite markers, are comprehensively discussed below and schematically presented in Fig. 2.

Various carbohydrate metabolites were significantly elevated following the marathon and can be ascribed to gluconeogenic influx (MacLaren and Morton, 2012) and a reduced insulin secretion (Richter *et al.*, 1992), which is typically induced by an initial depletion of glucose and glycogen stores occurring approximately 90 min after the start of a marathon (exercise intensity dependent) at a VO_{2max} >75% (Stellingwerff, 2012). The reduced insulin concentrations temporarily inactivate insulin-dependent glucose uptake systems (i.e. GLUT 4 transporters and glucokinase) (Salway, 2012), preventing glucose absorption into cells. This could ascribe the post-marathon elevations in serum glucose concentrations and various other associated metabolites, including glucaric acid (Żółtaszek *et al.*, 2008) and mannose (Hu *et al.*, 2016) as well as the elevated concentrations of gluconeogenesis-associated metabolites, i.e. myo-inositol (Eisenberg and Parthasarathy, 1987), erythritol (synthesized via erythrose-4-phosphate in pentose pathway) (Hootman *et al.*, 2017), glycerol and glyceric acid (Salway, 2012; Wadman *et al.*, 1976). Furthermore, the elevated concentrations of pyruvic acid observed in the post-marathon samples were anticipated since it is an end-product of the glycolysis pathway which feeds into the TCA cycle for further ATP production (Salway, 2012). This is confirmed by the accumulation of various TCA

cycle intermediates such as α-ketoglutaric acid, succinic acid, citric acid, fumaric acid and malic acid (Qiang, 2015), which also indicate the accumulation of circulating NADH/$FADH_2$ molecules (Esterhuizen *et al.*, 2017) as a result of a saturated ETC activity. It is also important to mention that many of the aforementioned carbohydrate metabolites, along with elevated concentrations of mannose (Hu *et al.*, 2016), sorbose (Guzik and Stachowicz, 2016), mannitol (McNutt, 2000), tagatofuranose (Kroger *et al.*, 2006), and threonic acid (an ascorbic acid derivative) (Simpson and Ortwerth, 2000), are well-known constituents of fruits/fruit juices, vegetables/vegetable juices, peanuts, energy bars, energy drinks and various other ergogenic aids consumed by the athletes during the course of the marathon (Jeukendrup, 2011; Pfeiffer *et al.*, 2012).

The reduction in intracellular glucose due to the aforementioned cellular uptake inhibition is known to activate lipolysis of free and adipose tissue-bound triacylglycerol (TAG) (MacLaren and Morton, 2012) as alternative fuel substrates. This is substantiated by the elevated concentrations of serum glycerol, monopalmitin and various free fatty acids (lauric acid, palmitic acid, palmitoleic acid, 11-eicosenoic acid, 11,14-eicosadienoic acid, myristoleic acid, α-linolenic acid, 5-dodecenoic acid, linoleic acid and oleic acid) (Kujala *et al.*, 2013; Lewis *et al.*, 2010; Peake *et al.*, 2014; Waśkiewicz *et al.*, 2012). Additionally, accumulated 3-hydroxy acids (β-hydroxyhexanoic acid) and 3-keto acids (β-hydroxy-α,β-didehydrosebacic acid) are indications of a saturated β-oxidation pathway, ascribed to the inhibition of the rate-limiting enzyme, β-hydroxyacyl dehydrogenase, which is pursued by 3-ketoacyl-CoA thiolase. This saturated β-oxidation pathway results in the catabolism of the accumulated fatty acids via α-oxidation (Roe and Ding, s.a.), thus substantiating the elevated concentrations of α-hydroxyoctanoic acid (C_8; an α-oxidation intermediate) and the odd-chain fatty acids (OCFA; tridecanoic acid [C_{13}], pentadecanoic acid [C_{15}], heptadecanoic acid [C_{17}] and 10-heptadecenoic acid [$C_{17:1}$]) detected in the post-marathon serum. It should however be mentioned that these OCFA may also arise from elevated dietary intake (Jenkins *et al.*, 2017) and/or autophagy of various cellular constituents during extensive energy-requiring states (Singh and Cuervo, 2011). Nevertheless, irrespective of their origins, these OCFAs are ultimately catabolized to propionyl-CoA (Pfeuffer and Jaudszus, 2016), hence the elevated β-hydroxypropionic acid observed in the post-

marathon serum. The elevated concentrations of β-hydroxybutyric acid and acetoacetic acid are anticipated, as these are alternative fuel substrates for the brain (Cahill and Vech, 2003) and skeletal muscles (Holloszy and Coyle, 1984) in hypoglycemic states, and could also be an indication of an imbalanced redox state (Esterhuizen *et al.*, 2017; Salway, 2012). Furthermore, the post-marathon elevations of malonic acid typically indicate the accumulation of malonyl-CoA, which is a long-chain fatty acid (LCFA) transport inhibitor (Salway, 2012) and could therefore be an additional reason for the increased cytosolic LCFAs (palmitic acid, palmitoleic acid, 11-eicosenoic acid, 11,14-eicosadienoic acid, myristoleic acid, α-linolenic acid, linoleic acid and oleic acid).

Most amino acids are catabolized into TCA cycle substrates via propionyl-CoA, succinyl-CoA, pyruvic acid or acetyl-CoA, depending on the specific amino acid (Salway, 2012). Reduced concentrations of amino acids (serine, glycine, alanine, aspartic acid, phenylalanine, tyrosine, threonine and methionine) and altered amino acid-associated metabolite concentrations (dimethylglycine (Holm *et al.*, 2003), pyroglutamic acid (Kumar and Bachhawat, 2012), indole-3-acetic acid (Salway, 2012) and glutaric acid (Peake, 2016)) were detected in the post-marathon serum, which indicates amino acid catabolism during the marathon. Furthermore, elevated concentrations of α-hydroxybutyric acid (an intermediate in the threonine/methionine pathway), further supports the aforementioned NADH:NAD^+ imbalance, ketoacidosis, reduced insulin secretion and impaired glucose absorption (Gall *et al.*, 2010). Additionally, elevated *p*-hydroxyphenylacetic acid and *p*-hydroxyphenyllactic acid could be indicative of mild liver injury/damage (Ghoraba *et al.*, 2014; Liebich and Pickert, 1985), which concur with previous findings (Jastrzebski *et al.*, 2015; Lippi *et al.*, 2011).

In accordance with previous findings (Pechlivanis *et al.*, 2010), reduced serum concentrations of the branched-chain amino acids (BCAAs) isoleucine (0.05 vs 0.02 µg/mL, p=0.018), valine and leucine were detected in the post-marathon samples, while their catabolism intermediates i.e. α-ketoisovaleric acid, β-hydroxyisobutyric acid (valine metabolites) and α-ethylhyrdracrylic acid (allo-isoleucine metabolism) (Wendel *et al.*, 1989) were significantly elevated. Allo-isoleucine catabolism intermediates may serve as alternative substrates in the valine catabolism pathway (Ryan, 2015),

however, since the valine catabolism intermediates remained elevated in the post-marathon serum it can be deducted that allo-isoleucine is instead catabolized to α-ethylhydracrylic acid (Korman *et al.*, 2005) via α-keto-β-methylvaleric acid (explaining its reduced concentrations post-marathon) with the subsequent production of $FADH_2$ (Korman *et al.*, 2005). The elevated concentrations of β-hydroxyisovaleric acid detected post-marathon is typically associated with ketone rich environments (as observed in the current investigation), resulting from increased leucine catabolism and the subsequent isovaleryl-CoA production (Mock *et al.*, 2011). This metabolite is also a well-known constituent of athlete supplementation (Brioche *et al.*, 2016) and could be elevated due to dietary ingestion during the marathon. Furthermore, reduced concentrations of these BCAAs, in particular leucine, results in mammalian target of rapamycin complex 1 inhibition (Laplante and Sabatini, 2009), which in turn activates various catabolic processes such as autophagy (of organelle and plasma membrane constituents) to release additional embedded fuel substrates (Singh and Cuervo, 2011). Mammalian target of rapamycin complex 1 (mTOR1) inhibition can also be induced by other factors including elevated 5'-AMP-activated protein kinase during energy deprivation, reduced oxygen levels, reduced essential amino acids and inflammation (Laplante and Sabatini, 2009), all of which are associated with endurance races and evidently occur in the marathon athletes investigated in this study. Considering that autophagy contributes to elevated amounts of cellular debris, the aforementioned accumulation of fatty acids and especially the OCFAs, in the post-marathon serum may also be explained by the autophagosomal degradation of phospholipids, sphingolipids (Kishimoto *et al.*, 1973; Maes *et al.*, 1996) and phytosphingosines (Kitamura *et al.*, 2017; Kondo *et al.*, 2014) found in cell and organelle membranes. Additionally, the elevated concentrations of squalene and 5-pregnene-3β,20α-diol in the post-marathon serum suggest cholesterol degradation (Charlton-Menys and Durrington, 2007; Salway, 2012), further supporting the activation of autophagy as the latter is a common constituent in cell membranes (Salway, 2012). Elevated 5-pregnene-3β,20α-diol (also known as 20α-dihydropregnenolone) post-marathon also indicates steroid metabolism activation via cholesterol catabolism as it is produced by the reduction of pregnenolone via 20-hydroxysteroid dehydrogenase (Ebner *et al.*, 2006). Pregnenolone is a precursor for the production of aldosterone and cortisol, the latter of which stimulates lipolysis, gluconeogenesis and protein catabolism (Salway, 2012).

Furthermore, a significant decrease in α-aminomalonic acid concentrations was observed in the post-marathon serum samples. Although the precise origin of this metabolite is unknown, it has been associated with abnormal protein oxidation, macrophage accumulation, non-essential amino acid oxidation via reactive oxygen species and pulmonary arterial hypertension indication (Bujak *et al.*, 2016).

The microbiome plays a crucial role in many essential metabolic processes required during strenuous exercise, including the regulation of energy metabolism, oxidative stress and inflammatory response pathways (Mach and Fuster-Botella, 2017). To this end, various microbial-associated metabolites (i.e. tagatofuranose, talofuranose, ethyl-α-D-glucopyranoside, arabitol, indole-3-propionic acid and D-rhamnose) were significantly altered in the post-marathon samples. Most of these metabolites are synthesized from host carbohydrate intermediates, either via catabolism or fermentation processes (arabitol: a sugar alcohol produced form arabinose/glucose (Kumdam *et al.*, 2014); and ethyl-α-D-glucopyranoside: an ethnolysis product from glucose (Hu *et al.*, 2013)). Indole-3-propionic acid is the deamination product of tryptophan, produced by gut microbes (e.g. *Clostridium sporogenes*) (Wikoff *et al.*, 2009) and further supports the notion of reduced amino acids. The reduced concentrations of D-rhamnose, a component of most Gram-positive bacterial cell walls (Mistou *et al.*, 2016), contradict the above-mentioned microbial product elevations. Since the particular nature of this reduction following completion of the marathon is unclear, it warrants future investigation.

In addition to these endogenously and microbially produced metabolites, elevated concentrations of ibuprofen were also observed post-marathon. Ibuprofen is a well-known non-steroidal, anti-inflammatory drug (Nieman *et al.*, 2006) commonly used by athletes for preventing muscle damage, muscle soreness and inflammation and was most likely consumed by some athletes prior to/during the marathon (Mcanulty *et al.*, 2007).

5. CONCLUSION

The results of this metabolomics investigation suggest that the body utilizes various fuel substrate pathways to comply with the high energy demands required during the marathon, including catabolism

of carbohydrates, lipids (β-oxidation and α-oxidation) and amino acids, as well as activation of ketogenesis and autophagy via mTOR1 inhibition. Considering the results of the current investigation as well as previous literature, the possible cascade of events contributing to this metabolic "snapshot" could be summarized as follows: (a) A proposed initial reduction in carbohydrate catabolism and glucose uptake via the insulin-dependent transporters lead to glycolysis dysregulation, ketogenesis activation and increased serum glucose. (b) A metabolic shift towards fatty acid utilization (from either endogenous or dietary TAGs) is induced, which (c) overwhelms/saturates the β-oxidation pathway, resulting in the α-oxidation of fatty acids. (d) Amino acids (from either endogenous or dietary protein catabolism) are also used as alternative fuel substrates, resulting in (e) mTOR1 inhibition and autophagy as the body desperately tries to generate the necessary fuel substrates to comply with the energy demand. (f) Lastly, various metabolic processes are activated to reduce oxidative stress and regulate/correct the redox imbalance.

Possible limitations of this study include human genotype/phenotype variation (an inevitable confounder) and the uncontrolled dietary intake of the athletes during the marathon. However, convincing athletes to deviate from their individualized supplementation protocols would be extremely difficult, if not impossible. Validation using a larger sample cohort could further substantiate the current findings. Nonetheless, these findings indicate the extensive metabolic changes induced by the marathon perturbation. Possible future prospects could be to investigate the effects of supplementing with amino acids, pre- and probiotics, and β-hydroxyisovaleric acid as a means of improving aerobic exercise performance, reduce skeletal muscle and liver damage, and enhance recovery.

6. FOOTNOTES

6.1 Author contribution

The concept and study were designed by DTL, ZS, GH, TC, KMK and EJS; samples were acquired from the Northumbria University in collaboration with GH, TC, KMK and EMS. ZS was responsible for manuscript drafting, data analysis and interpretation, the latter of which was assisted by DTL, LL and LJM. LL, LJM and DTL were involved in repeated manuscript reviewing, of which LL was greatly

involved with structural (format) editing. All of the authors revised and approved the final version of this manuscript.

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6.3 Funding statement

The authors have no specific funding to report.

6.4 Data availability statement

The current analysis is part of a larger study consisting of multiple aims which are being drafted into various manuscripts. Considering this, the datasets generated from this investigation are not publically available, but can be acquired from the corresponding author on reasonable request. The authors declare that all the results included within this study has been presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation.

7. COMPLIANCE WITH ETHICAL STANDARDS

7.1 Conflict of interest

The authors declare that there are no conflicts of interest, and that this manuscript, and the work described therein, is unpublished and has not been submitted for publication elsewhere.

7.2 Ethical approval

Ethical approval for this investigation, conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines, was obtained from the Research Ethics Committee of the Faculty of Health and Life Sciences at the Northumbria University in Newcastle upon

Tyne, UK (reference number: HLSTC120716). Informed consent was obtained from all individuals included in the study.

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9. TABLES

Table 1 Summary of the participant demographical information

Participant demographical information	Average ± Standard deviation
Age (years)	41 ± 12
Pre-marathon athlete weight (kg)	71.3 ± 10.1
Post-marathon athlete weight (kg)	69.2 ± 9.7
Marathon experience (years)	9 ± 8
Marathon experience (races)	16 ± 29
Finishing time (hh:mm:ss)	04:19:09 ± 00:49:01

Table 2 The significant serum metabolite markers best describing the variation between the pre- and post-marathon groups, listed alphabetically

Metabolite name (PubChem ID)	Pre-marathon athletes: Concentration (µg/ml) (Standard deviation)	Post-marathon athletes: Concentration (µg/ml) (Standard deviation)	Wilcoxon Signed Rank test (p value)	Wilcoxon Signed Rank test (ω value)	PCA (Power)
α-Aminomalonic acid (100714)	0.046 (0.022)	0.025 (0.009)	$3.1x10^{-4}$	0.458	0.031
α-Ethylhydracrylic acid (188979)	0.002 (0.001)	0.005 (0.002)	$1.3x10^{-6}$	0.615	0.025
α-Hydroxybutyric acid (11266)	0.019 (0.011)	0.045 (0.023)	$2.8x10^{-6}$	0.595	0.018
α-Hydroxyoctanoic acid (94180)	$8.7x10^{-5}$ ($5.3x10^{-5}$)	$1.2x10^{-4}$ ($6.3x10^{-5}$)	0.001	0.413	0.003
α-Ketoglutaric acid (51)	$2.9x10^{-4}$ ($2.1x10^{-4}$)	$4.5x10^{-4}$ ($1.8x10^{-4}$)	$1.1x10^{-4}$	0.490	0.009
α-Ketoisovaleric acid (5204641)	0.002 (0.001)	0.003 (0.001)	$1.7x10^{-4}$	0.478	0.017
α-Keto-β-methylvaleric acid (47)	0.009 (0.003)	0.007 (0.002)	0.005	0.358	0.025
α-Linolenic acid (5280934)	$1.6x10^{-4}$ ($8.1x10^{-5}$)	$4.5x10^{-4}$ ($2.6x10^{-4}$)	$2.1x10^{-6}$	0.602	0.021
β-Hydroxybutyric acid (441)	0.021 (0.019)	0.215 (0.161)	$1.2x10^{-6}$	0.617	0.030
β-Hydroxyhexanoic acid (151492)	$3.2x10^{-4}$ ($1.6x10^{-5}$)	$8.9x10^{-4}$ ($3.1x10^{-4}$)	$2.1x10^{-6}$	0.602	0.033
β-Hydroxyisobutyric acid (87)	$2.9x10^{-5}$ ($4.9x10^{-5}$)	$2.9x10^{-4}$ ($1.4x10^{-4}$)	$1.2x10^{-6}$	0.617	0.034
β-Hydroxyisovaleric acid (69362)	0.002 (0.001)	0.002 (0.001)	0.011	0.321	0.009

β-Hydroxypropionic acid (68152)	0.003 (0.001)	0.004 (0.001)	0.007	0.343	0.013
β-Hydroxy-α,β-didehydrosebacic acid (5366445)	0.005 (0.002)	0.006 (0.002)	0.009	0.333	0.013
5-Dodecenoic acid (5312377)	$1.3x10^{-4}$ ($2.8x10^{-5}$)	0.002 (0.001)	$1.2x10^{-6}$	0.617	0.037
5-Pregnene-3β,20α-diol (312224064)	$3.0x10^{-4}$ ($2.7x10^{-4}$)	$6.1x10^{-4}$ ($4.3x10^{-4}$)	$3.4x10^{-6}$	0.590	0.007
10-Heptadecenoic acid (86289714)	0.001 (0.001)	0.003 (0.001)	$1.2x10^{-6}$	0.617	0.030
11-Eicosenoic acid (142770)	0.001 (0.001)	0.004 (0.001)	$1.3x10^{-6}$	0.615	0.033
11,14-Eicosadienoic acid (3208)	0.002 (0.001)	0.003 (0.001)	$9.3x10^{-4}$	0.421	0.008
Acetoacetic acid (96)	$7.5x10^{-5}$ ($5.4x10^{-5}$)	$2.1x10^{-4}$ ($1.5x10^{-4}$)	0.002	0.393	0.014
Alanine (5950)	$4.2x10^{-4}$ ($2.9x10^{-4}$)	$2.6x10^{-4}$ ($1.5x10^{-4}$)	0.004	0.368	0.003
Arabitol (439255)	0.004 (0.001)	0.005 (0.002)	$9.9x10^{-4}$	0.418	0.006
Aspartic acid (5960)	0.020 (0.012)	0.013 (0.007)	0.014	0.311	0.019
Citric acid (311)	$9.6x10^{-4}$ ($4.5x10^{-4}$)	0.002 (0.001)	$8.9x10^{-5}$	0.498	0.012
Dimethylglycine (673)	0.023 (0.013)	0.017 (0.008)	0.011	0.324	0.008
D-Rhamnose (5460029)	0.001 (0.001)	0.001 (0.001)	$1.1x10^{-2}$	0.324	0.006
Erythritol (222285)	0.007 (0.002)	0.012 (0.004)	$1.6x10^{-6}$	0.610	0.016
Ethyl-α-D-glucopyranoside (91733361)	0.169 (0.085)	0.213 (0.094)	0.008	0.336	0.003
Fumaric acid (444972)	0.005 (0.002)	0.007 (0.002)	$7.5x10^{-4}$	0.428	0.018
Glucaric acid (33037)	$4.1x10^{-4}$ ($1.8x10^{-4}$)	0.001 (0.001)	0.002	0.391	0.010
Glucose (5793)	0.001 (0.001)	0.003 (0.005)	$8.7x10^{-6}$	0.565	0.025
Glutaric acid (743)	$3.1x10^{-4}$ ($1.9x10^{-4}$)	$4.7x10^{-4}$ ($2.8x10^{-4}$)	0.009	0.331	0.009
Glyceric acid (752)	0.005 (0.002)	0.008 (0.003)	$6.9x10^{-5}$	0.505	0.015
Glycerol (753)	0.088 (0.042)	0.447 (0.143)	$1.2x10^{-6}$	0.617	0.041
Glycine (750)	0.042 (0.019)	0.026 (0.013)	$7.5x10^{-4}$	0.428	0.024
Heptadecanoic acid (10465)	0.004 (0.001)	0.006 (0.001)	$1.2x10^{-6}$	0.617	0.020
Ibuprofen (3672)	$7.5x10^{-6}$ ($1.8x10^{-5}$)	0.004 (0.013)	$6.4x10^{-5}$	0.508	0.011
Indole-3-propionic acid (3744)	0.002 (0.001)	0.001 (0.001)	$1.8x10^{-4}$	0.475	0.004
Indole-3-acetic acid (802)	0.003 (0.002)	0.002 (0.001)	$8.1x10^{-4}$	0.426	0.021
Lauric acid (3893)	0.011 (0.003)	0.020 (0.005)	$1.2x10^{-6}$	0.617	0.026

Leucine (6106)	0.130 (0.087)	0.064 (0.044)	0.002	0.398	0.022
Linoleic acid (5280450)	0.124 (0.050)	0.168 (0.055)	$4.9x10^{-4}$	0.443	0.006
Malic acid (525)	0.004 (0.002)	0.007 (0.003)	$3.1x10^{-6}$	0.592	0.018
Malonic acid (867)	$3.1x10^{-4}$ ($1.2x10^{-4}$)	$4.1x10^{-4}$ ($1.7x10^{-4}$)	0.005	0.353	0.006
Mannitol (6251)	0.004 (0.004)	0.007 (0.010)	0.006	0.348	0.004
Mannose (18950)	$3.2x10^{-4}$ ($4.3x10^{-4}$)	0.003 (0.005)	$5.5x10^{-6}$	0.577	0.024
Methionine (6137)	0.006 (0.004)	0.003 (0.002)	$6.9x10^{-4}$	0.431	0.017
Myo-inositol (892)	0.031 (0.030)	0.045 (0.035)	$8.1x10^{-4}$	0.426	0.011
Monopalmitin (14900)	$3.9x10^{-4}$ ($2.1x10^{-4}$)	$6.7x10^{-4}$ ($3.6x10^{-4}$)	0.002	0.401	0.011
Myristoleic acid (5281119)	0.001 (0.001)	0.006 (0.002)	$1.2x10^{-6}$	0.617	0.037
Oleic acid (445639)	0.127 (0.072)	0.511 (0.196)	$1.3x10^{-6}$	0.615	0.022
Palmitic acid (985)	0.423 (0.124)	0.633 (0.128)	$1.6x10^{-5}$	0.548	0.016
Palmitoleic acid (445638)	0.009 (0.008)	0.042 (0.022)	$7.2x10^{-6}$	0.570	0.026
Pentadecanoic acid (13849)	0.004 (0.002)	0.008 (0.002)	$1.2x10^{-6}$	0.617	0.022
Phenylalanine (6140)	0.030 (0.015)	0.020 (0.009)	0.002	0.393	0.021
p-Hydroxyphenylacetic acid (127)	0.002 (0.001)	0.003 (0.002)	0.001	0.408	0.008
p-Hydroxyphenyllactic acid (9378)	0.003 (0.002)	0.005 (0.002)	$2.9x10^{-4}$	0.460	0.014
Pyroglutamic acid (7405)	0.060 (0.014)	0.050 (0.023)	$2.3x10^{-4}$	0.468	0.009
Pyruvic acid (1060)	0.013 (0.009)	0.024 (0.012)	$7.5x10^{-4}$	0.428	0.016
Serine (5951)	0.033 (0.024)	0.018 (0.011)	0.014	0.314	0.021
Sorbose (439192)	0.015 (0.006)	0.022(0.01)	$6.1\ x10^{-4}$	0.436	0.008
Squalene (638072)	0.002 (0.001)	0.003 (0.004)	0.006	0.351	0.011
Succinic acid (1110)	0.011 (0.008)	0.016 (0.010)	$5.5x10^{-6}$	0.577	0.006
Tagatofuranose (12306016)	0.101 (0.058)	0.190 (0.122)	$3.9x10^{-4}$	0.450	0.012
Talofuranose (15560229)	0.147 (0.050)	0.253 (0.090)	$2.3x10^{-5}$	0.538	0.011
Threonic acid (5460407)	0.011 (0.004)	0.016 (0.005)	$1.1x10^{-5}$	0.557	0.025
Threonine (6288)	0.021 (0.014)	0.011 (0.007)	0.003	0.381	0.017
Tridecanoic acid (12530)	$2.4x10^{-4}$ ($1.0x10^{-4}$)	$3.8x10^{-4}$ ($1.7x10^{-4}$)	$9.5x10^{-6}$	0.562	0.010
Tyrosine (6057)	0.019 (0.008)	0.014 (0.006)	0.007	0.343	0.018
Valine (6287)	0.188 (0.115)	0.094 (0.061)	$9.3x10^{-4}$	0.421	0.027

Abbreviations: PCA: Principle component analysis

10. FIGURE LEDGENDS

Fig. 1 Principle component analysis scores plot showing clear differentiation of the serum samples of marathon athletes before (denoted by circles) and after (denoted by squares) the completion of a marathon. The variance accounted for are indicated in parenthesis. Abbreviations: PC: principle component

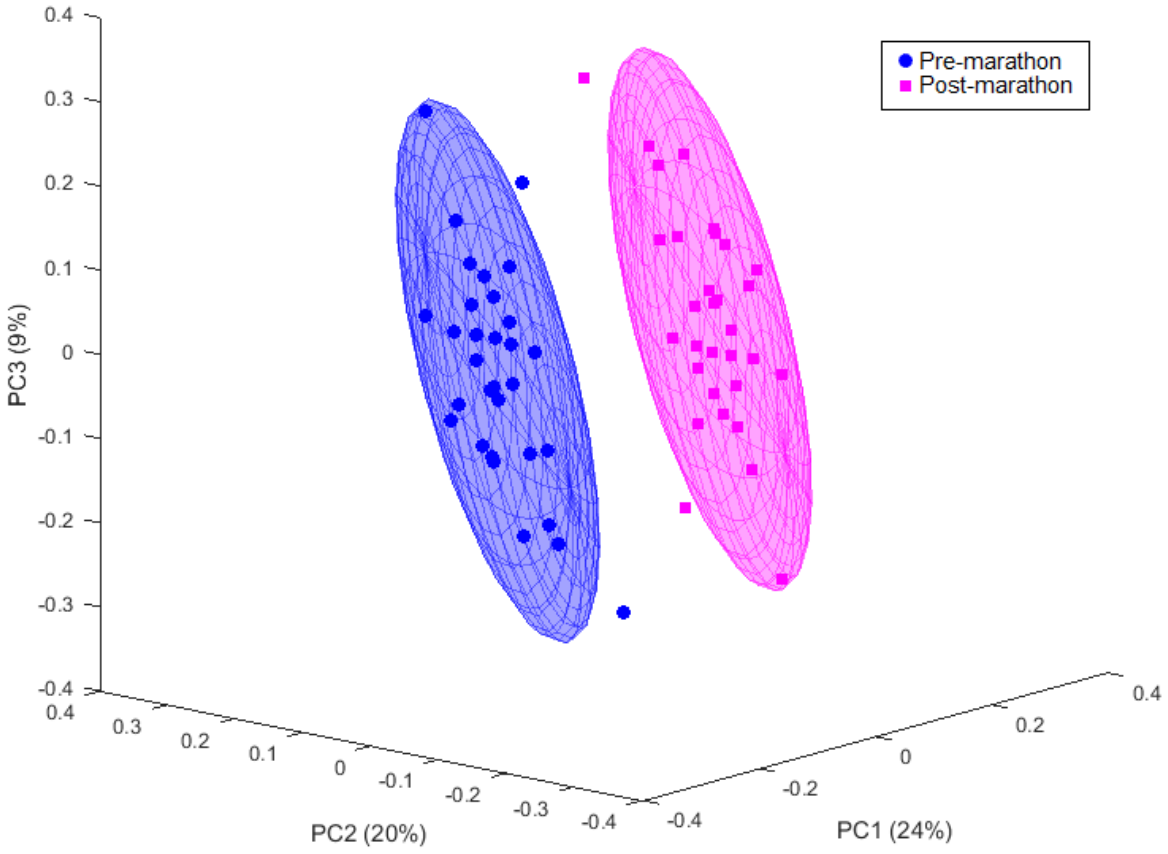

Fig. 2 A schematic representation of the altered serum metabolome induced by a marathon. The altered metabolites are either donated as increased (↑) or decreased (↓) relative to the pre-marathon group. Abbreviations: FAD: flavin adenine dinucleotide, FADH: flavin adenine dinucleotide + hydrogen, NAD: nicotinamide adenine dinucleotide, NADH: nicotinamide adenine dinucleotide + hydrogen, ATP: adenosine triphosphate, ADP: adenosine diphosphate